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PREPARATION AND CHARACTERIZATION OF PHOSPHOLIPID-DEPLETED CHLOROPLASTS

OSAMU HIRAYAMA and TAKASHI NOMOTOBORI

Department of Agriculture, Shimane University, Matsue 690 (Japan)

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Summary

Spinach class II chloroplasts were treated with snake venom phospholipase A_2 in the presence of bovine serum albumin, and separated by sucrose-density centrifugation. The treatment yielded phospholipid-depleted chloroplasts which had lost 82.6% of the original phospholipids. About 20% of the phospholipids of chloroplasts were resistant to enzyme attack. These results suggest that phospholipids exist in two states in chloroplast membranes.

In spite of considerable phospholipid depletion, the chloroplast preparations retained a large portion of their photoactivities, i.e. light-induced electron transport, light-induced H^+ uptake, and light-induced shrinkage. However, cyclic photophosphorylation was significantly affected with the phospholipid removal.

Introduction

Lipid depletion of chloroplasts is a valuable approach to an understanding of lipid functions in chloroplasts. Many workers [1—7] have investigated the effects of lipid removal lyophilized chloroplasts by solvent extraction and reconstitution of activities by adding back the extracted lipids. It was demonstrated that plastoquinones as well as β-carotene play an important role in the photochemical reactions in chloroplasts [1,4,5]. However, it seems that the procedures used were not suitable for the removal of polar lipids, e.g. galactolipids and phospholipids, because lipid removal by solvent extraction is too drastic and easily causes an irreversible disintegration of chloroplast membranes.

Abbreviations: DCIP, 2,6-dichlorophenylindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMBQ, 2,5-dimethyl-*p*-benzoquinone.

In the present study, we have devised a simple procedure for removing lipids from chloroplasts without a secondary disturbance in membrane structure. The procedure involves two processes; treatment of chloroplasts with a lipolytic enzyme in the presence of bovine serum albumin and subsequent separation by sucrose-density centrifugation. The properties of the phospholipid-depleted chloroplasts were studied, and the functions of phospholipids in chloroplasts are discussed.

Materials and Methods

Chloroplast preparation

Spinach leaves were homogenized in a medium containing 0.35 M NaCl, 10 mM Tris · HCl (pH 8.0), and 5 mM $MgCl_2$. The pellets which sedimented between 200 and 2000 × *g* were washed with a medium containing 10 mM Tris · HCl (pH 8.0) and 5 mM $MgCl_2$.

Phospholipase A_2 preparation and enzyme assay

Phospholipase A_2 was extracted and purified from snake venom of Habu. The preparation was confirmed to have no proteolytic activity. Phospholipase activity was assayed by determining the fatty acid released according to the method previously reported [8,9].

Enzymic treatment of chloroplasts

Standard reaction mixture (1 ml) for treatment with phospholipase A_2, contained 50 mM Tricine/KOH (pH 8.0), 0.4 M sucrose, 0.1 mM $CaCl_2$, 10 mM NaCl, 5 mM $MgCl_2$, chloroplast (1 mg chlorophyll), and the enzyme (50 μg protein) with or without bovine serum albumin (30 mg). The mixture was incubated at 20 or 30°C with shaking. After incubation for 20 min, the mixture was cooled rapidly to 0°C, and then used for measurements of activity, lipid analysis, and sucrose-density centrifugation.

Fixation of chloroplasts

Chloroplasts were fixed essentially by the method of Hallier and Park [10] with partial modification. Glutaraldehyde solution (10%) containing 0.4 M sucrose and 10 mM Tris · HCl (pH 7.0) was added to an equal volume of chloroplast suspension (1 mg chlorophyll/ml) containing the sucrose buffer. The suspension was stirred thoroughly at 0°C for 30 min, and then diluted with the sucrose buffer and centrifuged for 30 min at 15 000 × g. The precipitate was washed twice with the sucrose buffer.

Measurements

DCIP photoreduction ($H_2O \rightarrow$ DCIP) was measured spectrophotometrically at 610 nm. The reaction mixture (3 ml) contained 50 mM Tricine/KOH (pH 8.0), 10 mM NaCl, 0.1 mM DCIP, and chloroplasts (30 μg chlorophyll). Methyl viologen-mediated O_2 uptake was measured polarographically with a YSI 4004 Clark oxygen electrode. The $H_2O \rightarrow$ methyl viologen assay mixtre (3 ml) contained 50 mM Tricine/KOH (pH 8.0), 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose, 200 μM NaN_3, 0.1 mM methyl viologen, and chloroplasts (30 μg

chlorophyll). In the $DCIPH_2$ → methyl viologen assay, the mixture (3 ml) contained 0.1 mM DCIP, 50 mM asorbic acid, and 10 μM DCMU in addition to the mixture for the H_2O → methyl viologen assay. The rate of O_2 evolution (H_2O → DMBQ) was measured polarographically with the Clark oxygen electrode described above. The reaction mixture (3 ml) contained 50 mM Tricine/KOH (pH 8.0), 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose, 200 μM NaN_3, 1 mM DMBQ, and chloroplasts (30 μg chlorophyll). All the reactions were carried out under white light illumination (40 000 lux) from a projector lamp at 25°C.

Light-induced H^+ uptake was evaluated by measurements of pH change on the basis of the procedure reported by Dilley [11]. The reaction mixture (4 ml) contained 100 mM KCl, 5 mM $MgCl_2$, 50 μM phenazine methosulfate, and chloroplasts (80 μg chlorophyll). The pH change was observed at an initial pH of 6. Cyclic photophosphorylation was also assayed by a similar determination of pH change according to the method reported by Nishimura et al. [12]. The reaction mixture (4 ml) was similar to that of the light-induced H^+ uptake with the following additions: 1 mM ADP and 1 mM K_2HPO_4, initial pH 8.0. 90° light scattering at 550 nm was estimated essentially according to the method described by Murakami and Packer [13]. The reaction mixture contained 50 mM Tricine/NaOH (pH 7.3), 150 mM sodium acetate, 20 μM phenazine methosulfate, and chloroplasts (10 μg chlorophyll/ml). The measuring light was filtered at 550 nm with an interference filter, Toshiba KL-55, and the intensity in the dark before illumination of the suspension was adjusted to read 100% on the chart paper. Chloroplasts were illuminated by red light (>650 nm) through a color filter glass, Toshiba V-R 65.

Lipid analysis

The chloroplast suspension (1 ml) was extracted twice with *n*-butanol (1 ml). The extracts were evaporated under reduced pressure, and dissolved in chloroform (1 ml). The resulting lipid solution in chloroform was then washed with 0.9% NaCl, and used for silica gel thin-layer chromatography analysis or silica gel micro-column analysis. Lipid identification and quantitation by thin-layer chromatography were carried out according to the method previously reported [14]. Micro-column analysis using a small glass tube (6 × 20 mm) was applied to a separation of glycolipid and phospholipid classes; each lipid fraction was hydrolyzed and estimated by determining the released fatty acid on the basis of the method previously reported [8].

Results

Extent of phospholipid degradation in the treatment of chloroplasts with phospholipase A_2

Fig. 1 shows the time course of phospholipid degradation in chloroplasts when unfixed or glutaraldehyde-fixed chloroplasts were treated with phospholipase A_2. In the figure, the hydrolysis rate of phospholipids was expressed in terms of phosphatidylglycerol which is a major phospholipid component (183 nmol/mg chlorophyll) in chloroplasts. In most cases, a large portion of the phopholipids was hydrolyzed in the early stage of incubation (for 10 min), and the residual phospholipids were resistant to incubation even during a time of

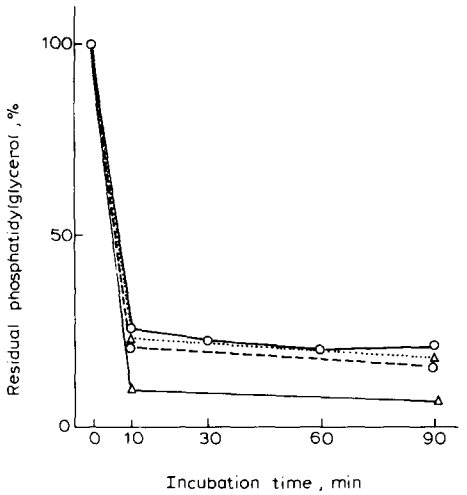

Fig. 1. Enzymatic degradation of phospholipids in glutaraldehyde-fixed and unfixed chloroplasts. Spinach class II chloroplasts were fixed with glutaraldehyde as described in Materials and Methods. The fixed and unfixed chloroplasts (1 mg chlorophyll) were treated with snake venom phospholipase A_2 at 30°C in the presence or the absence of bovine serum albumin. Phospholipid degradation rates were followed by a combination of microcolumn and thin-layer chromatographic analysis using densitometry, and expressed as the residual contant of phosphatidylglycerol (%). ○———○, glutaraldehyde-fixed chloroplast, without bovine serum albumin in the enzymic reaction; ○- - - - -○, fixed chloroplasts, with bovine serum albumin; △· · · · ·△, unfixed chloroplasts, with bovine serum albumin; △———△, unfixed chlorplasts, without bovine serum albumin.

incubation of 80 min. Unfixed chloroplasts, when treated in the absence of bovine serum albumin, exhibited a higher rate of hydrolysis (around 90%), and the rate decreased to around 80% by the addition of bovine serum albumin and also by the fixation of chloroplasts. These repressions of hydrolysis rate are possibly due to the grana stacking, which was stimulated with bovine serum albumin [15] and maintained by fixation without a swelling of membranes.

Preparation of the phospholipid-depleted chloroplasts

Chloroplasts (3 mg chlorophyll) were treated with the phospholipase A_2 under the same condition as described in Methods with bovine serum albumin (30 mg/ml). After incubation at 20°C for 20 min, the mixture was cooled rapidly to 0°C, and then separated by sucrose-density gradient centrifugation. A discontinuous sucrose gradient of three layers was prepared by pipetting 12 ml of 1.6 M, 10 ml of 1.2 M, and 5 ml of 0.4 M sucrose in 10 mM Tris · HCl buffer (pH 7.5) containing 20 mM NaCl and 5 mM $MgCl_2$. The reaction mixture was layered on top of the gradient. The sample was centrifuged for 60 min at 80 000 × *g*. A main green band of chloroplasts was found on the interface between 1.2 M and 1.6 M sucrose layer, and most bovine serum albumin added and the lipid-hydrolyzing products (e.g. free fatty acid, lysophosphatidylcholine etc.) remained in the top layer. The resulting chloroplast preparation (phospholipid-depleted chloroplasts) had lost 82.6% of phospholipids on the basis of the control chloroplast which was subjected to the same procedure but without the enzyme. As shown in Table I, the residual phospholipids in the preparation were phosphatidylglycerol (24.0% of total phosphatidylglycerol) and phosphatidylcholine (7.9% of total phosphatidylcholine).

TABLE I

EFFECTS OF THE PHOSPHOLIPID REMOVAL ON THE PHOTOCHEMICAL FUNCTIONS OF CHLOROPLASTS

Phospholipids of chloroplasts were removed through enzymic treatment and subsequent separation by sucrose-density centrifugation as in Methods. The resulting phospholipid-depleted chloroplasts were assayed and compared with the control chloroplasts which were prepared by the same procedure without the enzyme. Analysis of phospholipids and assay of the photochemical functions of chloroplasts were carried out as Materials and Methods.

Lipid contents and chloroplast functions	Chloroplast preparation		
	Control	Phospholipid-depleted	
			Percentage of control
Phospholipid content *	310	54	17.4
Phosphatidylglycerol	183	44	24.0
Phosphatidylcholine	127	10	7.9
Light-induced electron flow **			
O_2 evolution ($H_2O \rightarrow$ DMBQ)	124	107	86
$DCIPH_2 \rightarrow$ methyl viologen	212	196	93
$H_2O \rightarrow$ methyl viologen	97	85	88
Light-induced H^+ uptake ***	1619	1349	83
Cyclic photophosphorylation †	181	117	64
90° light-scattering increase ††	132	111	84

* nmol/mg chlorophyll.
** μmol O_2 evolved or consumed/mg chlorophyll per h.
*** nequiv. H^+ accumulated/mg chlorophyll.
† μmol ATP formed/mg chlorophyll per h.
†† Percentage of the initial value.

Characterization of the phospholipid-depleted chloroplasts

The preparation of phospholipid-depleted chloroplasts was examined for photochemical functions. The results are summarized in Table I, compared with the control chloroplasts. It is suprising that, in spite of phospholipid removal to the extent of 82.6%, the preparation retained a large portion of the original photoactivities. The rates of electron transports were 86% and more of those of the control chloroplasts. The extent of H^+ uptake and the photoshrinkage activity measured by 90° light-scattering, were also maintained at a similar or slightly lower level. But, a higher loss of activity was found in cyclic photophosphorylation, suggesting that CF_1 was significantly affected in the enzyme treatment.

Discussion

The enzymic degradation of phospholipids in chloroplasts (in Fig. 1), shows that the phospholipids exist in two states in the membrane. In one state (about 20% of the total phospholipids), the phospholipids are not attacked by phospholipase A_2. In the other state the phospholipids are easily hydrolyzed with the lipolytic enzyme. It appears that the proportion of the two types changes to some degree depending on the overall structure of the membranes.

Recently, on the basis of a study with lipid spin label, Jost et al. [16] have demonstrated that there were two types of the membrane lipids, i.e. the

boundary lipids associated with protein and the fluid lipids in lipid bilayer. Robinson and Capaldi [17] have further defined three types of the phospholipids: fluid, boundary and tightly bound, on the basis of studies with cytochrome *c* oxidase. If this is the case in chloroplast membranes, the residual phospholipids in the present experiment could be assigned probably to the tightly bound lipids, because the lipids have been reported to be resistant to phospholipase action [18], and most of the enzyme-digested phospholipids could be assigned to the fluid lipids.

Characterization of the phospholipid-depleted chloroplasts suggests that a large portion of phospholipids, presumably the fluid lipids, could be removed without strong effects on the photochemical functions of chloroplasts, i.e. light-induced electron transport, light-induced H^+ uptake, and light-induced shrinkage (in Table I). This could be interpreted to suggest that the residual phospholipids (presumably the tightly bound lipids), rather than the enzyme-digested lipids, play an important role in the photoactivities.

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